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Blue Dextran: Influence on Chromatographic Profile and Immunoreactivity of Human Follicle-Stimulating Hormone (HFSH) (38852)

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(Introduced by F. C. Bartter)

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We have previously reported that when iodinated hFSH is chromatographed over long Sephadex G-100 columns, the radioactivity elutes in three peaks which we attributed to aggregates of FSH, monomers of FSH, and subunits of FSH, respectively (1). The highest immunoreactivity, and the highest testicular receptor-binding affinity were found in the aggregate peak (1, 2). Talas Midgley, and Jaffe have previously pointed out that Blue Dextran (BD) influences the elution pattern of FSH from Sephadex G-100 (3). We report studies that show conclusively that the presence of FSH "aggregates" is consequent upon the use of BD, and we show that BD has the surprising effect of increasing the apparent immunoreactivity of the ^{125}I-hFSH which elutes in the region of the void volume.

Materials and Methods. Highly purified human pituitary FSH (LER-1575C) and rabbit anti-hFSH antiserum were generously supplied by the National Pituitary Agency, Bethesda, Md. Goat anti-rabbit IgG was obtained from Miles-Yeda Ltd., Rehovot, Israel. Human FSH was iodinated by a modification of the Chloramine T method of Greenwood *et al.* (4). The hFSH radioimmunoassay was performed by a modification of the double-antibody method of Odell *et al.* (5). Lot LER-1575C was used for iodination, and also provided the unlabeled standard. Chromatography on Sephadex G-100 was performed with a long 90 × 2.5-cm column of descending type. The elution buffer was 0.01 *M* phosphate–0.14*M* saline buffer with 0.1% bovine serum albumin (0.1% BSA-PBS). The buffer flow rate was 20 ml/hr and when BD was used, 2 mg was routinely added. Four-milliliter fractions were collected in a refrigerated automatic fraction collector.

Results and Discussion. A. Elution profile of ^{125}I-hFSH. A representative profile is shown in the lower panel of Fig. 1. FSH was iodinated on 19 April 1974 and free iodine was removed by running the iodination mixture over a short Sephadex G-75 column. The iodinated FSH (SpAc 50 μCi/μg) was divided into aliquots containing about 30 × 10^6 cpm each, and these were stored at −20 C. One tube was chromatographed immediately on Sephadex G-100, without addition of BD, and the elution pattern of radioactivity is shown in Column I Fig. 1(a), lower panel. A single large peak is present, with $K_{av} = 0.20$, which is compatible with FSH monomer.

On April 28 an aliquot of ^{125}I-FSH was thawed, 2 mg of BD was added, and the mixture was applied to the Sephadex column (Column II). Two peaks of radioactivity were present: Peak 1 in the region of the void volume (V_0), and Peak 2 in the "FSH monomer" region.[1]

On the following day (April 29) a further aliquot of ^{125}I-FSH was chromatographed without the addition of BD (Column III) in order to evaluate whether freezing and thawing of ^{125}I-FSH might be responsible for the production of Peak I. The elution pattern of radioactivity is shown in Fig. 1 (b), lower panel. Only Peak 2 appeared, and the pattern was almost identical to that of Column I, Fig. 1.

On May 1 2 mg BD was added to Fraction 52 of Column III (i.e., a fraction from the FSH monomer peak) and the mixture was rechromatographed on Sephadex G-100 (Column IV). The elution pattern of radioactivity, shown in Fig. 1 (d), lower panel,

[1] A peak of radioactivity in the V_0 region is referred to as Peak I; any peak in the FSH monomer region is referred to as Peak 2 even when an earlier peak is not present.

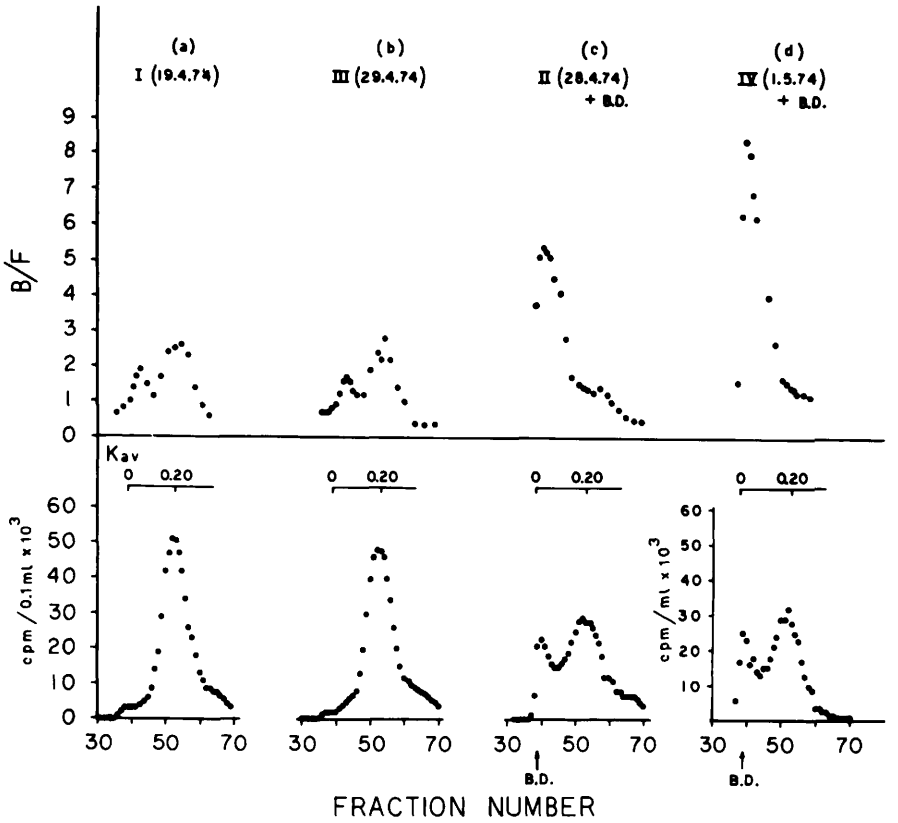

FIG. 1. Behavior of ^{125}I-FSH on Sephadex G-100 when chromatographed in the absence of Blue Dextran (Columns I and III) and in the presence of Blue Dextran (Columns II and IV). Lower panel: profile of radioactivity. Upper panel: Immunoreactivity of aliquots of 4000 cpm. A detailed description of Columns I to IV appears in the text.

contains Peak 1 and Peak 2, and is similar to that of Column II.

B. *Immunoreactivity of ^{125}I-FSH in Columns I–IV.* We have examined the immunoreactivity of the various fractions by incubating aliquots each containing 4000 cpm with rabbit anti-hFSH antiserum (final dilution 1:3,200) in a final volume of 0.8 ml for 18 hr at room temperature. After addition of "second antibody" (goat anti-rabbit IgG) the tubes were incubated for a further 2 hr at 35° before centrifugation and separation of bound (B) from free (F) radioactivity. The immunoreactivity of the fractions of each column was examined on the day after the chromatography was performed.

Results are shown in Fig. 1 (a–d), upper panel. In Columns I and III (to which BD was not added) the highest B/F, about 2.6, was observed at a K_{av} = 0.21, which corresponds to Peak 2 of radioactivity. A small shoulder of immunoreactivity was also observed on the ascending limb of Peak 2. In columns II and IV (to which BD was added) maximal values of B/F (5.4 and 8.4) were coincident with Peak 1 of radioactivity: maximal B/F in the region of Peak 2 was only about 1.4, that is, less than in the same region of Columns I and III. The appearance of Peak 1 is clearly related to the presence of BD and, under these circumstances, this peak of radioactivity exhibits maximal immunoreactivity under standard immunoassay conditions.

To further compare immunoreactivity of ^{125}I-FSH from Peaks 1 and 2, five standard curves were prepared with exactly the same conditions for each, save for the ^{125}I-FSH tracer: Peak I of Columns II and IV, and Peak 2 of Columns II, III, and IV. LER-

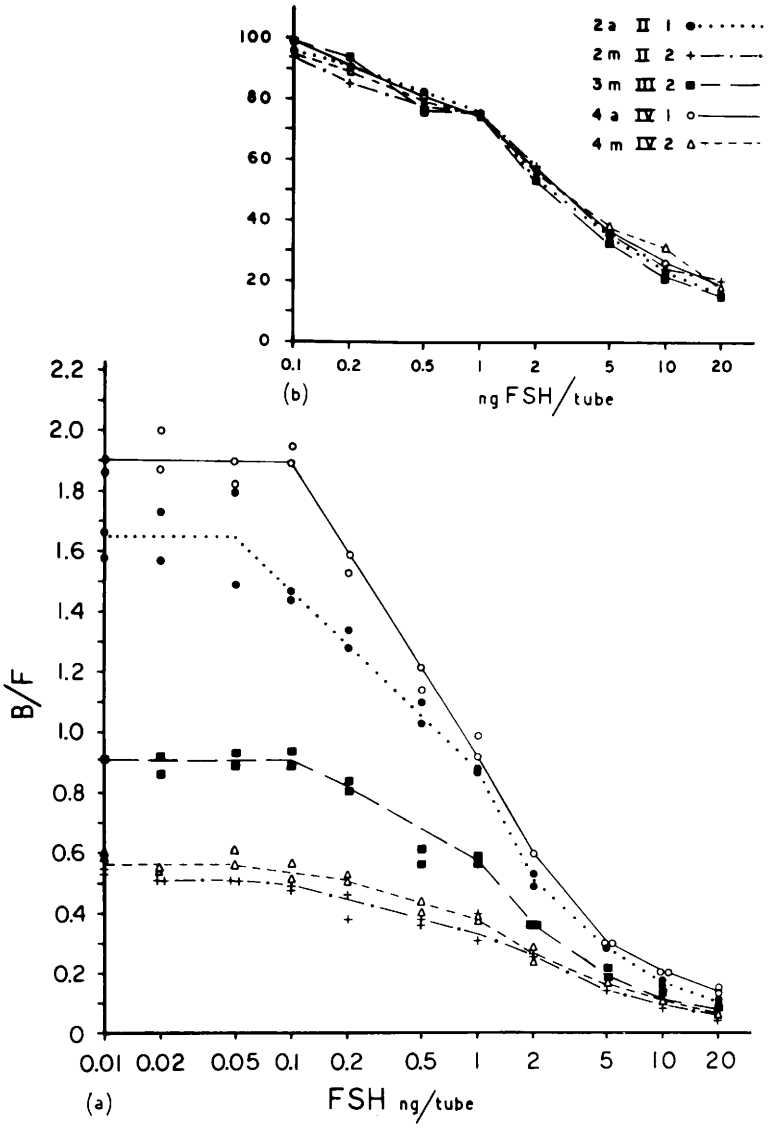

FIG. 2. Standard curves for hFSH radioimmunoassay performed under identical conditions but employing five different tracers. II_1 and II_2 refer to tracers from Column II, Peaks 1 and 2, respectively; similarly, III_2 refers to Peak 2 of Column III, IV_1 and IV_2 refer to Peaks 1 and 2, respectively, from Column IV. Figure 2(a) plots the data as B/F versus log FSH (ng/tube); 2(b) replots the same data with the ordinate showing binding as a percentage of binding in the presence of tracer only.

1575C was employed as unlabeled standard, and the final dilution of anti-hFSH antiserum was 1:16,000.

The five plots are shown in Fig. 2 (a). The striking differences in maximal binding of tracer among the five curves corresponds closely to measurements of immunoreactivity shown in Fig. 1: the greatest binding was observed in curves with tracer from Peak 1, and the lowest in curves using Peak 2 as tracer from columns to which BD had been added. When the curves are replotted with the ordinate showing percentage of maximal binding (Fig. 2(b)) all five curves become virtually superimposable.

C. *Effect of BD on the elution pattern of*

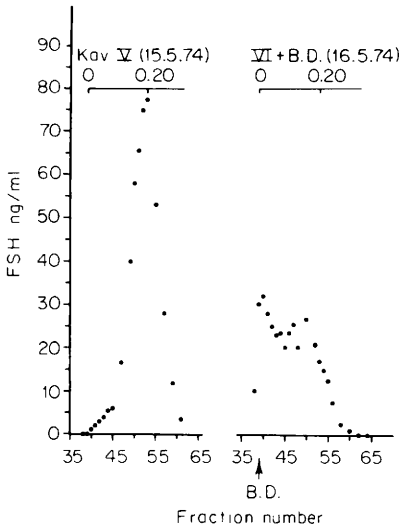

FIG. 3. Profile of uniodinated hFSH chromatographed on Sephadex G-100. Column V: No BD present; Column VI: BD present.

uniodinated FSH after Sephadex G-100 chromatography. On two occasions, 4 μg of LER-1575C was run on the 90 × 2.5-cm Sephadex G-100 column, either without BD (Column V) or with 2 mg of BD (Column VI). The FSH concentrations in the various fractions were measured by radioimmunoassay. Results are shown in Fig. 3. The influence of BD on the elution pattern of uniodinated FSH corresponds to that observed with iodinated FSH.

D. *Rechromatography of ^{125}I-FSH from Peak 1 of Column II after storage at 4°.* Fraction 39–42 of Column II (April 28) were pooled. Four milliliters from this pool were run on the same Sephadex G-100 column on June 2. The pattern of elution is shown in Fig. 4 (a). Most of the radioactivity now appears in the Peak 2 region. We have previously reported similar findings (1). When exactly the same procedure was repeated but with the addition of 2 mg BD, most of the radioactivity eluted in the Peak 1 area, and only a minor fraction eluted in the Peak 2 area (Fig. 4(b)).

We have thus confirmed the findings of Talas *et al.* (3) that hFSH, chromatographed over Sephadex G-100 in the presence of BD, elutes, in part, in the region of the void volume. Our present experiments are in full agreement with our original studies, but we

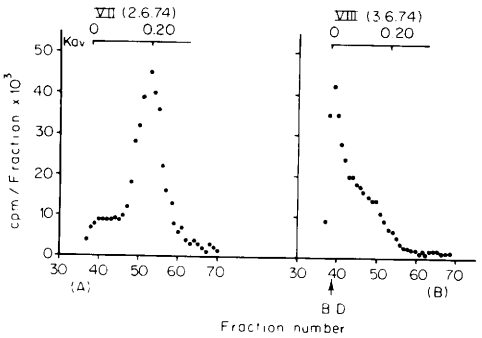

FIG. 4. Rechromatography of Fractions 39–42 of Column II. Column VII: No BD present; VIII: BD added.

had failed initially to recognize the influence of Blue Dextran on the elution profiles of FSH. High-molecular-weight aggregates of FSH, based on Bio-Gel chromatography, have also been noted by Gray (6). From the published figures appearing in this paper, it appears that Blue Dextran was routinely employed as a column marker. However, it remains possible that factors other than Blue Dextran may play a role in formation of FSH aggregates.

We have demonstrated that BD has consistent effects on the immunoreactivity of labeled hFSH: the higher binding to antibody is found in Peak 1, in the region of the void volume, relative to that observed in Peak 2. In the absence of BD, maximal immunoreactivity is observed in Peak 2 which corresponds to the elution volume of FSH "monomer".

We can offer several possible explanations for enhancement of immunoreactivity of the ^{125}I-FSH associated with BD. Conceivably BD may have some direct influence on the antigen–antibody binding reaction. Alternatively, we can hypothesize that several molecules of FSH, labeled and unlabeled, might be attached to a molecule of BD. An anti-hFSH molecule might need to bind only one of these FSH molecules, and not necessarily a labeled one, for the whole BD–FSH complex to be precipitated when the second antibody is added. Other explanations are possible. Practically we have found that for routine immunoassays use of tracer from Peak 1 gives good results after an 18 -to 20-hr incubation at room temperature (1). Fur-

thermore, the tracer FSH from Peak 1 not only exhibits higher immunoreactivity but also shows higher specific binding to rat testis particulate fraction than does Peak 2 (2). However, Fig. 2 clearly shows that, whereas tracer from Peak 1 gives a higher absolute B/F than does tracer from Peak 2, all five curves are superimposable when the ordinate is plotted as "percentage of bound radioactivity".

Summary. When FSH is chromatographed on G-100 Sephadex in the absence of Blue Dextran, it elutes as a single peak in the region of monomeric FSH. The addition of Blue Dextran causes a significant portion of FSH, iodinated or uniodinated, to elute in the region of the void volume. When standard curves for FSH are performed under identical conditions, with tracer ^{125}I-FSH from the void volume, and from the FSH monomer region, maximal immunoreactivity was significantly greater for the Blue Dextran-associated tracer.

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